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A molecular phylogeny of *Pseudocrangonyx* from Japan, including a new subterranean species (Crustacea, Amphipoda, Pseudocrangonyctidae)

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Abstract

A subterranean species of pseudocrangonyctid amphipod, *Pseudocrangonyx gudariensis* Tomikawa & Sato, **sp. n.**, is described from the spring-fed stream Gudari-numa in Hakkoda Mountains, Aomori Prefecture, northern Japan. *Pseudocrangonyx gudariensis* is morphologically similar to *P. coreanus* Uéno, 1966 and *P. febras* Sidorov, 2009 based on its relatively small body size, small number of articles of rami of pleopods, and urosomite 1 without basal setae. However, *P. gudariensis* is distinguished from those species based on the following characteristics: from *P. coreanus*, antenna 2 of female without calceoli, palmar margins of gnathopods 1 and 2 with distally notched robust setae, inner margin of inner ramus of uropod 2 with 4 robust setae, and basal part of inner ramus of uropod 2 without slender seta; and from *P. febras*, carpus of gnathopod 2 without serrate robust setae on posterodistal corners, peduncle of pleopods 1 and 2 with setae, and longer article 2 of uropod 3. Phylogenetic analyses using nuclear 28S rRNA and histone H3, and mitochondrial cytochrome *c* oxidase subunit I and 16S rRNA markers showed that *P. gudariensis* is placed among known *Pseudocrangonyx* Akatsuka and Komai, 1922 species. However, its exact phylogenetic position within the genus could not be determined. The polyphyly of the Japanese *Pseudocrangonyx* species indicates that multiple colonization events of *Pseudocrangonyx* ancestors to the Japanese Archipelago could have occurred. The reliability of the past *Pseudocrangonyx* records from Japan is briefly discussed.

Introduction

Amphipods that belong to the genus *Pseudocrangonyx* Akatsuka & Komai, 1922 inhabit subterranean waters of Japan, the Korean Peninsula, eastern China, and the Far East of Russia; this genus currently includes 20 species (Sidorov and Gontcharov 2013).

Pseudocrangonyx was originally established for the three Japanese subterranean species (Akatsuka and Komai 1922): *P. shikokunis* Akatsuka & Komai, 1922; *P. yezonis* Akatsuka & Komai, 1922; and *P. kyotonis* Akatsuka & Komai, 1922. The first species, *P. shikokunis*, which was subsequently designated as the type species by Barnard and Barnard (1983) by position precedence, was reported from Tokushima Prefecture in Shikoku, and Hyogo, Okayama, and Yamaguchi Prefectures in the western area of Honshu, Japan (Akatsuka and Komai 1922, Uéno 1927, 1933a, c, Tomikawa et al. 2008); *P. yezonis* was collected in Hokkaido and Akita Prefecture in the northern area of Honshu (Akatsuka and Komai 1922, Uéno 1933b, Matsuda 1954); and *P. kyotonis* has been recorded from Kyoto, Gifu, Shizuoka, and Shimane Prefectures in Honshu (Akatsuka and Komai 1922, Uéno 1927, 1971a, c, Nunomura 1975). Additionally, two continental *Pseudocrangonyx* species were reported from Japanese waters: *P. asiaticus* Uéno, 1934 from Tsushima Island (Uéno 1971b); and *P. coreanus* Uéno, 1966 from Tsushima Island and Shimane Prefec-

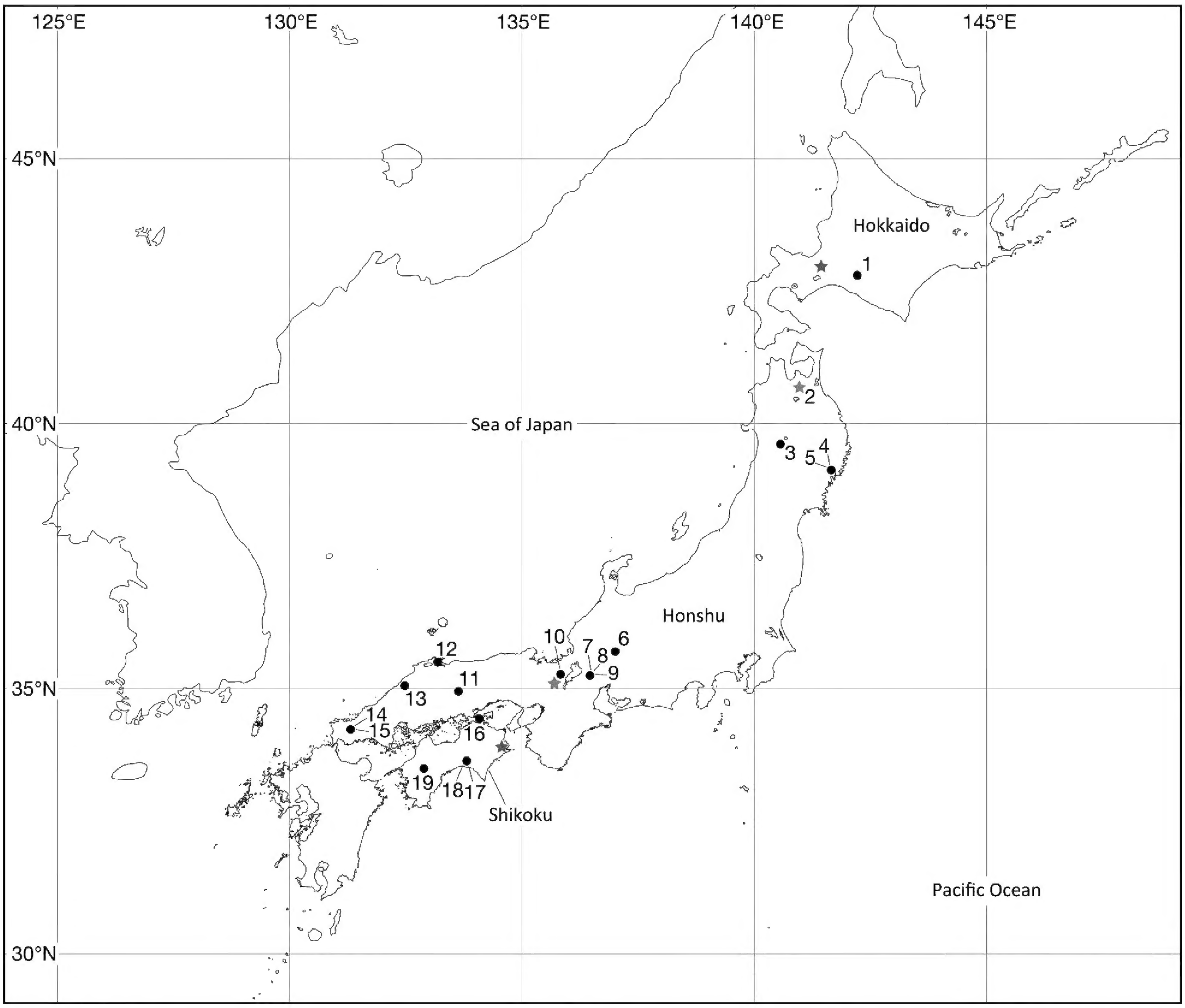

Figure 1. Map showing the collection localities of the specimens examined in this study and type localities of the known Japanese *Pseudocrangonyx* species. The closed circles indicate the localities of the referred materials used for the phylogenetic analyses. The star in red denotes the type locality of the new species; in purple, *P. shikokunis*; in blue, *P. kyotonis*; and in green, *P. yezonis*. Names of localities are shown in Table 1.

ture in the western area of Honshu (Uéno 1971b, Narahara et al. 2009).

Over recent decades, additional species have been described from the Far East of Russia (e.g., Sidorov and Gontcharov 2013). These results indicate that *Pseudocrangonyx* is highly diversified, and several species await description (Sidorov and Gontcharov 2013). Therefore, it is highly possible that there are also many undescribed species in the Japanese Archipelago.

During field surveys of the benthic invertebrate fauna in the spring-fed stream Gudari-numa in Hakkoda Mountains, Aomori Prefecture in the northern Honshu, two of the authors (AS and AO) and their colleagues collected several *Pseudocrangonyx* specimens. After careful examination of the materials, it was revealed that the collected *Pseudocrangonyx* amphipods represent an undescribed species. Thus, this new species is described herein. In addition, the phylogenetic position of the new species within *Pseudocrangonyx* was estimated using nuclear 28S rRNA and histone H3, and mitochondrial cytochrome *c* oxidase subunit I and 16S rRNA sequence data.

The taxonomic description was prepared by the first and third authors (KT and AS). The second author (TN) conducted the molecular analyses, the fourth author (YO) assisted in manuscript preparation, and the last author (AO) provided the material of the new species and conducted this study.

Material and methods

Sample

Specimens of *Pseudocrangonyx* species were collected from 14 localities in Hokkaido, Honshu, and Shikoku, Japan (Fig. 1). Most of specimens were collected by scooping various groundwater environments in caves with a fine-mesh hand-net. The Gudari-numa specimens were pumped up with 10–55 L of interstitial water at 21–57 cm

beneath the surface of gravelly bottom using a handmade core sampler, and then collected, or directly collected by scooping together with bottom gravels of the stream. Most of the specimens were fixed in approximately 10% formaldehyde solution but a few were in 99% ethanol.

Morphological observation

All appendages of the examined specimens of the undescribed species were dissected in 70% ethanol and mounted in gum-chloral medium on glass slides under a stereomicroscope (Olympus SZX7). Specimens were examined using a light microscope (Nikon Eclipse Ni) and illustrated with the aid of a camera lucida. The body length from the tip of the rostrum to the base of the telson was measured along the dorsal curvature to the nearest 0.1 mm. The nomenclature of the setal patterns on the mandibular palp follows Stock (1974). The specimens are deposited in the Tsukuba Collection Center of the National Museum of Nature and Science, Tokyo (NSMT) and the Zoological Collection of Kyoto University (KUZ).

PCR and DNA sequencing

The extraction of genomic DNA from appendage muscles of the *Pseudocrangonyx* materials preserved in 99% ethanol followed Tomikawa et al. (2014). Primer sets for the PCR and cycle sequencing (CS) reactions used in this study were as follows: for 28S rRNA (28S), 28F and 28R (PCR and CS) (Hou et al. 2007) with 28SF and 28SR (CS) (Tomikawa et al. 2012) as internal primers; for histone H3 (H3), H3aF and H3bR (PCR and CS) (Colgan et al. 1998); for cytochrome *c* oxidase subunit I (COI), LCO1490 and HCO2198 (PCR and CS) (Folmer et al. 1994), or jgLCO1490 and jgHCO2198 (Geller et al. 2013), respectively, with M13F and M13R tails (Messing 1983), used for PCR, and then M13F and M13R used as primers for CS, followed Raupach et al. (2015); for 16S rRNA (16S), 16STf (Macdonald III et al. 2005) and 16Sbr (Palumbi 1996; modified to correspond with "Fruit Fly") (PCR and CS).

The PCR reaction and DNA sequencing for a part of COI and 16S sequences followed Tomikawa et al. (2014); the PCR and CS reactions were performed using a PC-320 Thermal Cycler (ASTEC). Those for the other sequences were performed using the modified method outlined by Nakano (2012) and Tomikawa et al. (2016); reactions were performed using a T-100 Thermal Cycler (Bio-Rad). When using a PC-320 Thermal Cycler, the PCR mixtures were heated to 94°C for 7 min, followed by 35 cycles at 94°C (45 s), 42°C (1 min) and 72°C (1 min), and a final extension at 72°C (7 min). In the other reactions using a T-100, the PCR mixtures were heated to 94°C for 6 min, followed by 35 cycles at 94°C (10 s), 50°C for 28S and H3 or 48°C for COI and 16S (20 s each), and 72°C (1 min 24 s for 28S, 24 s for H3, and 42 s for COI and 16S), and a final extension at 72°C for 6 min. When using a PC-320, the CS conditions were 25 cycles at 96°C (10 s), 50°C (5 s) and 60°C (4 min). The sequencing mixtures for the other reactions were heated to 96°C for 2 min, followed by 40 cycles at 96°C (10 s), 50°C (5 s) and 60°C (36 s). The obtained sequences of a portion of COI and 16S were edited using MEGA6.03 (Tamura et al. 2013), and the reminders were assembled using DNA BASER (Heracle Biosoft S.R.L.). These DNA sequences were deposited with the International Nucleotide Sequence Database Collaboration (INSDC) through the DNA Data Bank of Japan (DDBJ) (Table 1).

Molecular phylogenetic analyses

Twenty-one published sequences were obtained from the INSDC for use in molecular phylogenetic analyses (Table 1). Eleven OTUs of the seven *Pseudocrangonyx* species, *P. febras* Sidorov, 2009, *P. holsingeri* Sidorov & Gontcharov, 2013, *P. korkishkoorum* Sidorov, 2006, *P. kseniae* Sidorov, 2012, *P. susanaensis* Labay, 1999, *P. sympatricus* Sidorov & Gontcharov, 2013, and *P. tiunovi* Sidorov & Gontcharov, 2013, distributed in the Russian Far East were included in the analyses along with the following three crangonyctoid amphipods as outgroup taxa: *Crangonyx floridanus* Bousfield, 1963, *Crymostygius thingvallensis* Kristjánsson & Svavarsson, 2004, and *Eocrangonyx primoryensis* Stock & Jo, 1990.

The phylogenetic position of the *Pseudocrangonyx* amphipod from the Gudari-numa Stream within the genus was estimated based on the gene fragments of 28S, H3, COI, and 16S sequences. The alignments of H3 and COI was trivial, as no indels were observed. The 28S, and 16S sequences were aligned using MAFFT v. 7.299b L-INS-i (Katoh and Standley 2013). The lengths of 28S, H3, COI and 16S sequence lengths were 1,480, 328, 658, and 431 bp, respectively.

Prior to construction a phylogenetic tree based on the concatenated sequences, maximum likelihood (ML) trees were constructed based on each of the 28S, COI, and 16S markers using RAxML v. 8.2.8 (Stamatakis 2014) with the substitution model set as GTRCAT, immediately after nonparametric bootstrapping (Felsenstein 1985) conducted with 1,000 replicates. Based on the three obtained ML trees (not shown), a 28S (HQ286019) and a COI (HQ286032) sequences of *C. thingvallensis* were removed from the dataset to prevent long branch attraction. Then, 28S sequences were re-aligned using MAFFT L-INS-i and refined with Gblocks Server v. 0.91b (Castresana 2000) with a default setting, and thus their final length was 980 bp. The concatenated sequences yielded 2,397 bp of alignment positions. One of the completely identical sequences (G1297 and G1298) was removed from the dataset using the "pgelimdupseq" command implemented in Phylogears v. 2.0.2014.03.08 (Tanabe 2008).

ML phylogenies were conducted using RAxML v. 8.2.8 with GTRCAT, immediately after nonparametric bootstrapping (BS) conducted with 1,000 replicates. The best fit-partitioning scheme for the ML analysis was identified with the Akaike information criterion (Akaike 1974) using PartitionFinder v. 1.1.1 (Lanfear et al. 2012) with the "all" algorithm: 28S/1st and 2nd positions of H3/3rd position of H3/1st position of COI/2nd position of COI/3rd position of COI/16S. BI and Bayesian poste-

Table 1. Samples used for the phylogenetic analyses. The information on the vouchers is accompanied by the collection localities and the INSDC accession numbers. Sequences marked with an asterisk were obtained for the first time in the present study. Acronym: NSMT, the Tsukuba Collection Center of the National Museum of Nature and Science, Tokyo. Identification sources: a, by the first author KT; b, Narahara et al. (2009); c, Nunomura (1975); d, Uéno (1927); e, Uéno (1971a).

#	Species	Voucher or isolate #	Loclaity	INSDC #			
				28S	Histone H3	COI	16S
Pseudocrangonyx							
1	*P. yezonis*[a]	G1280	Mukawa, Hokkaido	LC171518*	LC171520*	LC171519*	LC171517*
2	*Pseudocrangonyx* sp.	NSMT-Cr 24605	Aomori, Aomori	LC171498*	LC171500*	LC171499*	LC171497*
3	*P. yezonis*[a]	G1279	Daisen, Akita	LC171514*	LC171516*	LC171515*	LC171513*
4	*Pseudocrangonyx* sp.	G400	Ofunato, Iwate				LC171479*
5	*Pseudocrangonyx* sp.	G1281	Ofunato, Iwate				LC171521*
6	*P. kyotonis*[c]	G1297	Gujo, Gifu	LC171541*	LC171543*	LC171542*	LC171540*
6	*P. kyotonis*[c]	G1298	Gujo, Gifu	LC171545*	LC171547*	LC171546*	LC171544*
7	*Pseudocrangonyx* sp.	G404	Taga, Shiga	LC171488*	LC171489*		
8	*Pseudocrangonyx* sp.	G405	Taga, Shiga	LC171491*	LC171493*	LC171492*	LC171490*
9	*Pseudocrangonyx* sp.	G406	Taga, Shiga	LC171495*	LC171496*		LC171494*
10	*Pseudocrangonyx* sp.	G1282	Otsu, Shiga		LC171523*		LC171522*
11	*Pseudocrangonyx* sp.	G1283	Niimi, Okayama	LC171525*	LC171527*	LC171526*	LC171524*
12	*P. kyotonis*[e]	G402	Matsue, Shimane	LC171485*	LC171487*	LC171486*	LC171484*
13	*P. coreanus*[b]	G401	Ota, Shimane	LC171481*	LC171483*	LC171482*	LC171480*
14	*P. shikokunis*[d]	G1277	Mine, Yamaguchi	LC171506*	LC171508*	LC171507*	LC171505*
15	*Pseudocrangonyx* sp.	G1278	Mine, Yamaguchi	LC171510*	LC171512*	LC171511*	LC171509*
16	*Pseudocrangonyx* sp.	G1271	Takamatsu, Kagawa	LC171502*	LC171504*	LC171503*	LC171501*
17	*Pseudocrangonyx* sp.	G1295	Kami, Kochi	LC171533*	LC171535*	LC171534*	LC171532*
18	*Pseudocrangonyx* sp.	G1296	Kami, Kochi	LC171537*	LC171539*	LC171538*	LC171536*
19	*Pseudocrangonyx* sp.	G1294	Seiyo, Ehime	LC171529*	LC171531*	LC171530*	LC171528*
	P. febras					KF153114	
	P. holsingeri			KJ871679		KF153111	
	P. korkishkoorum	B1		KJ871678		KF153107	
	P. korkishkoorum	B2				KF153108	
	P. korkishkoorum	B3				KF153109	
	P. korkishkoorum	N1		KJ871676		KF153105	
	P. korkishkoorum	N2		KJ871677		KF153106	
	P. kseniae			KJ871675		KF153115	
	P. susanaensis					KF153113	
	P. sympatricus					KF153112	
	P. tiunovi			KJ871674		KF153110	
Outgroup							
	Crymostygius thingvallensis			HQ286019		HQ286032	HQ286009
	Eocrangonyx primoryensis						HQ286011
	Crangonyx floridanus	G1322	Chiba, Chiba	LC171549*		LC171550*	LC171548*

rior probabilities (PPs) were estimated suing MrBayes v. 3.2.6 (Ronquist et al. 2012). The best-fit partition scheme as well as models for each partition were selected based on the Bayesian information criterion (Schwarz 1978) using PartitionFinder with the "all" algorithm: for 28S, GTR+G; for the 1st and 2nd positions of H3, JC69; for the 3rd position of H3, K80+G; for the 1st position of COI, SYM+I; for the 2nd position of COI, HKY85+I+G; for the 3rd position of COI, GTR+I+G; and for 16S, GTR+I+G. Two independent runs of four Markov chains were conducted for 20 million generations, and the tree was sampled every 100 generations. The parameter estimates and convergence were checked using Tracer v. 1.6.0 (Rambaut and Drummond 2013) and the first 50,001 trees were discarded based on these results.

Results

Taxonomy

Pseudocrangonyx gudariensis Tomikawa & Sato, sp. n.

http://zoobank.org/A821F75A-4FCB-40F7-B8AE-48289A696725
New Japanese name: Gudarimekura-yokoebi
Figs 2–9

Type materials. Holotype: Male (3.9 mm), NSMT-Cr 24603, Gudari-numa Stream (40°40'21.2"N, 140°55'54.6"E, elev. 589 m), Komagome, Aomori, Aomori Prefecture, Japan, 4 June 2014, collected by A. Ohtaka. Paratypes: 1 female (3.1 mm), NSMT-Cr 24604, locality same as for holotype, 21 June 2015, collected by

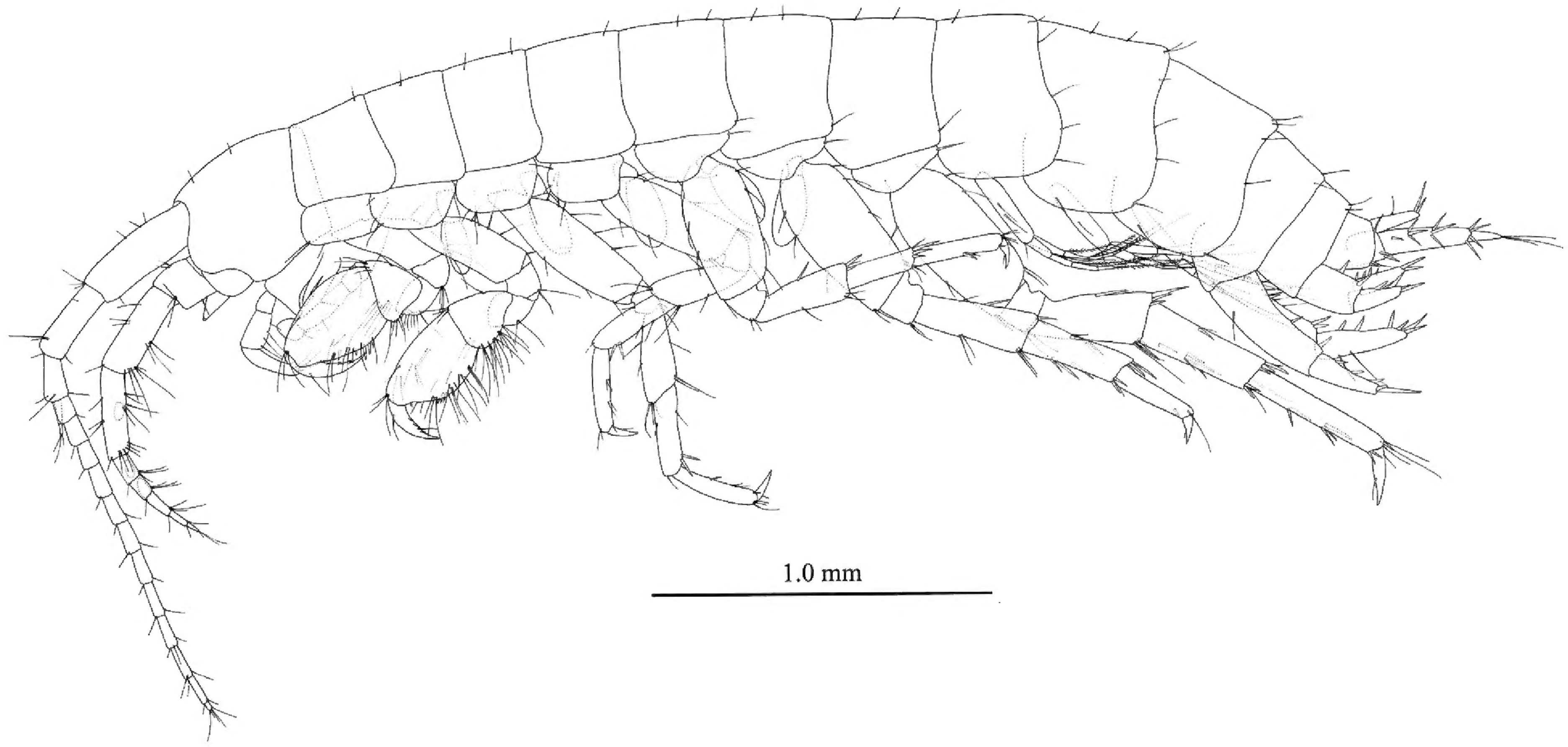

Figure 2. *Pseudocrangonyx gudariensis* Tomikawa & Sato, sp. n., holotype, male (3.9 mm), NSMT-Cr 24603. Habitus, lateral view.

A. Ohtaka; 1 female (4.4 mm), NSMT-Cr 24605, locality same as for holotype, 14 March 2015, collected by A. Ohtaka; 1 male (2.5 mm) and 3 females (1.4–2.8 mm), NSMT-Cr 24606, locality same as for holotype, 23 May 2015, collected by A. Ohtaka; 4 females (2.0–2.9 mm) KUZ Z1746, data same as for holotype, collected by A. Ohtaka.

Type locality. Japan, Aomori Prefecture: Aomori, Gudari-numa Stream (northern Honshu).

Description. *Male* [NSMT-Cr 24603, 3.9 mm]. Head (Fig. 2) with short dorsal setae; rostrum reduced; lateral cephalic lobe rounded; antennal sinus shallow with rounded angle; eyes absent. Pereonites 1–7 with short dorsal setae (Fig. 2); posterolateral margin of pereonites 5–7 with seta (Fig. 2). Dorsal margin of pleonites 1–3 with 5, 6, and 8 setae, respectively (Fig. 7I–K). Dorsal margin of urosomites 1 and 2 each with 4 setae (Fig. 7L, M), dorsal margin of urosomite 3 lacking setae (Fig. 7N). Posterior margin of epimeral plate 1 with 2 setae, posteroventral corner rounded with 1 seta (Fig. 7P); ventral and posterior margins of plate 2 with 1 and 2 setae, respectively, posteroventral corner rounded, with 1 seta (Fig. 7Q); ventral and posterior margins of plate 3 each with 1 seta, posteroventral corner rounded, with 1 seta (Fig. 7R).

Antenna 1 (Fig. 3A) 0.47 times as long as body length, peduncular articles 1 to 3 in length ratio of 1.0 : 0.6 : 0.4; accessory flagellum 2-articulate, terminal article with 3 setae; primary flagellum 12-articulate, 1 aesthetasc on some articles. Antenna 2 (Fig. 3B) 0.60 times as long as antenna 1; peduncular article 5 with calceolus; flagellum 0.55 times as long as peduncular articles 4 and 5 combined, consisting of 4 articles, first 2 of which with calceolus.

Upper lip (= labrum) (Fig. 3C) with rounded anterior margin, bearing fine setae. Mandibles (Fig. 3E, F) with left and right incisors both 5-dentate; left lacinia mobilis 4-dentate, right lacinia bifid, bearing many teeth; molar process triturative, molar of right mandible with accessory seta; accessory setal rows of left and right mandibles with 3 and 2 weakly pectinate setae, respectively; palp 3-articulate, article 3 with 1 A-, 4–5 D-, and 4 E-setae. Lower lip (Fig. 3D) with broad outer lobes, mandibular process of outer lobe rounded apically; inner lobes indistinct. Maxilla 1 (Fig. 3G) with inner and outer plates, and palp; inner plate subovate, its medial margin with 3 plumose setae; outer plate subrectangular with 7 serrate teeth apically; palp 2-articulate, longer than outer plate, article 1 lacking marginal setae, article 2 with 3 apical and 1 subapical robust setae. Maxilla 2 (Fig. 3H) with oblique inner row of 3 plumose setae on inner plate. Maxilliped (Fig. 4A–C) with inner and outer plates, and palp; inner plate (Fig. 4C) with 3 apical and 2 subapical robust setae; outer plate (Fig. 4B) with 1 apical plumose seta and 1 apical robust seta and 5 medial slender setae; palp (Fig. 4A) 4-articulate, medial margin of article 2 lined with setae, article 4 with nail.

Gnathopod 1 (= pereopod 1) (Fig. 4D, E) with subquadrate coxa, bearing setae on anterodistal corner of coxa, width 2.0 times as long as depth; anterior margin of basis bare, posterior margin of basis with 5 setae; posterodistal corner of carpus with slender setae, some weakly pectinate; propodus stout, subchelate, palmar margin with 6 medial and 5 lateral robust setae, some distally notched (Fig. 4E); posterior margin of dactylus dentate (Fig. 4E). Gnathopod 2 (= pereopod 2) (Fig. 4F, G) with subquadrate coxa bearing setae on its anterior margin and posterodistal corner, width 1.9 times as long as depth; anterior and posterior margins of basis with 0 and 5 setae, respectively; posterodistal corner of carpus with slender seta, some weakly pectinate; propodus stout, subchelate with 6 medial and 8 lateral robust setae along palmar margin, some

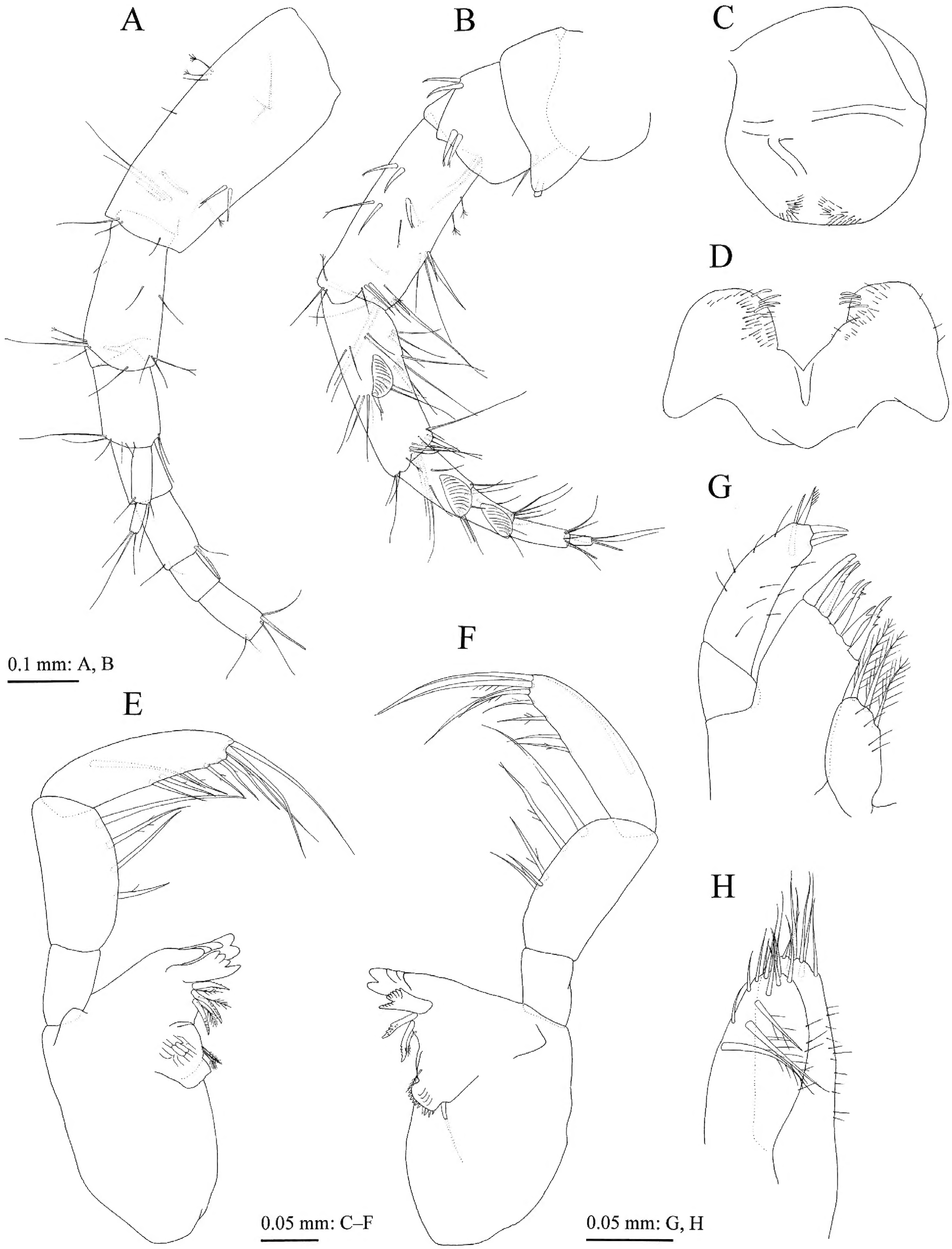

Figure 3. *Pseudocrangonyx gudariensis* Tomikawa & Sato, sp. n., holotype, male (3.9 mm), NSMT-Cr 24603. **A** antenna 1, medial view; **B** antenna 2, medial view; **C** upper lip, anterior view; **D** lower lip, ventral view; **E** left mandible, medial view; **F** right mandible, medial view; **G** maxilla 1, dorsal view; **H** maxilla 2, dorsal view.

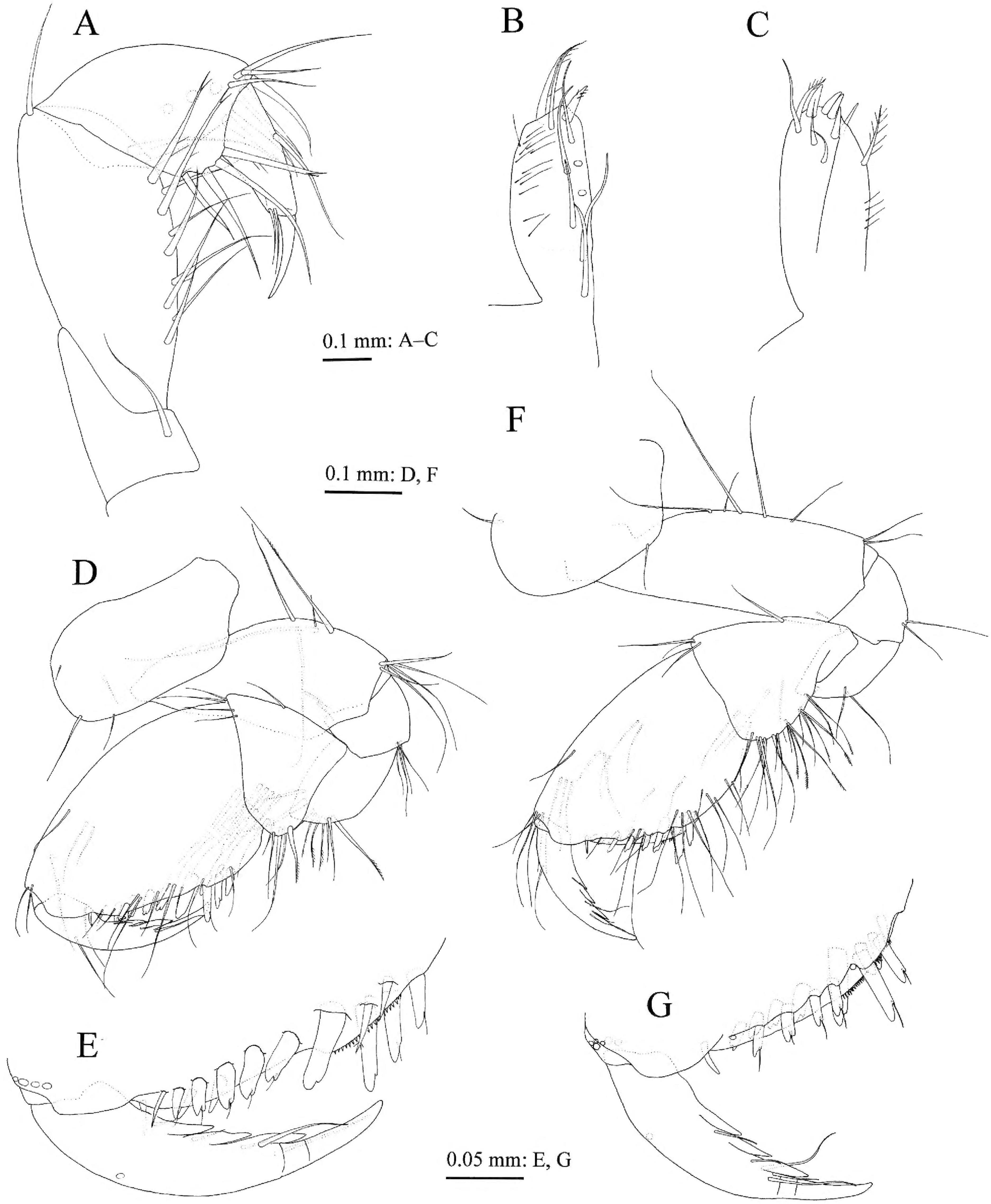

Figure 4. *Pseudocrangonyx gudariensis* Tomikawa & Sato, sp. n., holotype, male (3.9 mm), NSMT-Cr 24603. **A** maxilliped, dorsal view; **B** inner plate of maxilliped, dorsal view; **C** outer plate of maxilliped, dorsal view; **D** gnathopod 1, lateral view; **E** palmar margin of propodus and dactylus of gnathopod 1, medial view; **F** gnathopod 2, lateral view; **G** palmar margin of propodus and dactylus of gnathopod 2, medial view.

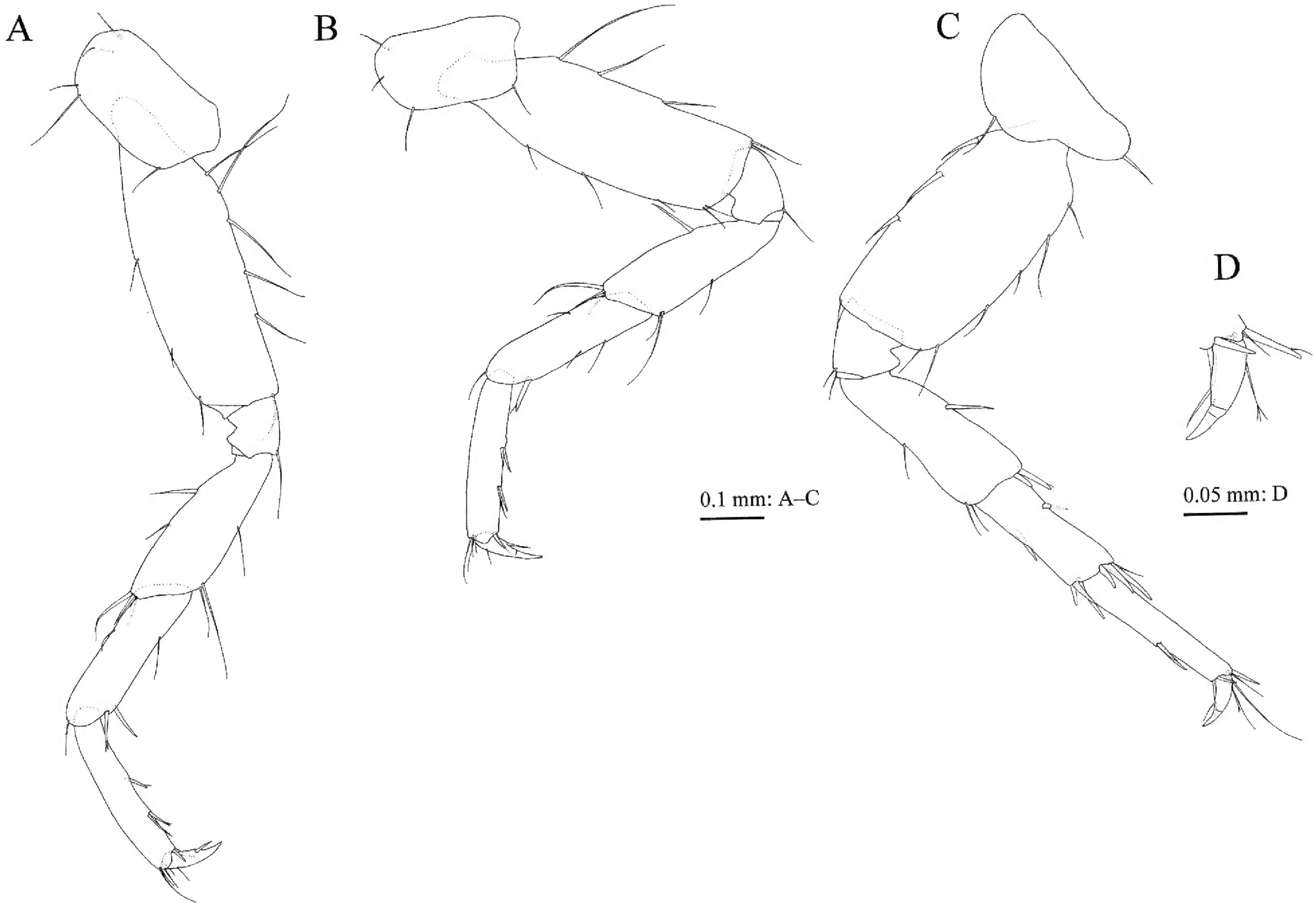

Figure 5. *Pseudocrangonyx gudariensis* Tomikawa & Sato, sp. n., holotype, male (3.9 mm), NSMT-Cr 24603. **A** pereopod 3, lateral view; **B** pereopod 4, lateral view; **C** pereopod 5, lateral view; **D** dactylus of pereopod 5, lateral view.

distally notched (Fig. 4G); posterior margin of dactylus dentate (Fig. 4G). Pereopod 3 (Fig. 5A) with subquadrate coxa bearing setae on its anterodistal and and posteroventral corners, width 1.8 times as long as depth; anterior and posterior margins of basis with 2 and 5 setae, respectively; merus, carpus, and propodus in length ratio of 1.0 : 1.0 : 0.9; posterior margin of dactylus with 1 seta. Pereopod 4 (Fig. 5B) with subquadrate coxa bearing setae on its anterior margin, anterodistal and posteroventral corners, width 1.8 times as long as depth; anterior and posterior margins of basis each with 3 setae; merus, carpus, and propodus in length ratio of 1.0 : 0.8 : 0.8; posterior margin of dactylus with 1 seta. Pereopod 5 (Fig. 5C, D) with weakly bilobed coxa bearing setae on anterior and posterior lobes; anterior and posterior margins of basis with 3 and 4 setae, respectively; merus, carpus, and propodus in length ratio of 1.0 : 0.8 : 0.9; posterior margin of dactylus with 2 setae (Fig. 5D). Pereopod 6 (Fig. 6A, B) with coxa bearing concave lower margin, posteroproximal corner with 1 seta; anterior and posterior margins of basis with 4 and 3 setae, respectively; merus, carpus, and propodus in length ratio of 1.0 : 0.9 : 1.1; posterior margin of dactylus with 2 setae (Fig. 6B). Pereopod 7 (Fig. 6C–E) with subtriangular coxa, bearing 1 seta on posteroproximal corner; anterior and posterior margins of basis each with 3 setae; merus, carpus, and propodus in length ratio of 1.0 : 1.1 : 1.2; posterior margin of dactylus with 2 setae (Fig. 6E).

Coxal gills (Fig. 2) on gnathopod 2 and pereopods 3–6; sternal gills absent.

Peduncles of pleopods 1 and 2 (Fig. 7A, C) with 2 and 1 setae on outer margins, respectively; peduncle of pleopod 3 (Fig. 7D) lacking marginal setae. Pleopods 1–3 each with paired retinacula (Fig. 7B), and lacking bifid setae (clothes-pin setae) on inner basal margin of inner ramus.

Uropod 1 (Fig. 7E) with basofacial slender seta on peduncle; inner ramus 0.70 times as long as peduncle, inner margin of former with 2 robust setae, outer margin bare, basal part with 3 slender setae; outer ramus 0.76 times as long as inner, its inner and outer margins with 0 and 2 robust setae, respectively. Uropod 2 (Fig. 7F, G) with inner and outer rami; inner ramus 0.90 times as long as peduncle, its inner margin with 4 robust setae, outer margin bare, distal part with 2 serrate and 4 simple robust setae and 1 slender seta (Fig. 7G); outer ramus 0.89 times as long as inner ramus, its outer margin with 1 robust seta. Uropod 3 (Fig. 7H) with peduncle 0.33 times as long as outer ramus, with 1 dorsal and 3 ventral robust setae; inner ramus absent; outer ramus 2-articulate, proximal article with robust setae, terminal article 0.36 times as long as proximal article, with 3 distal setae.

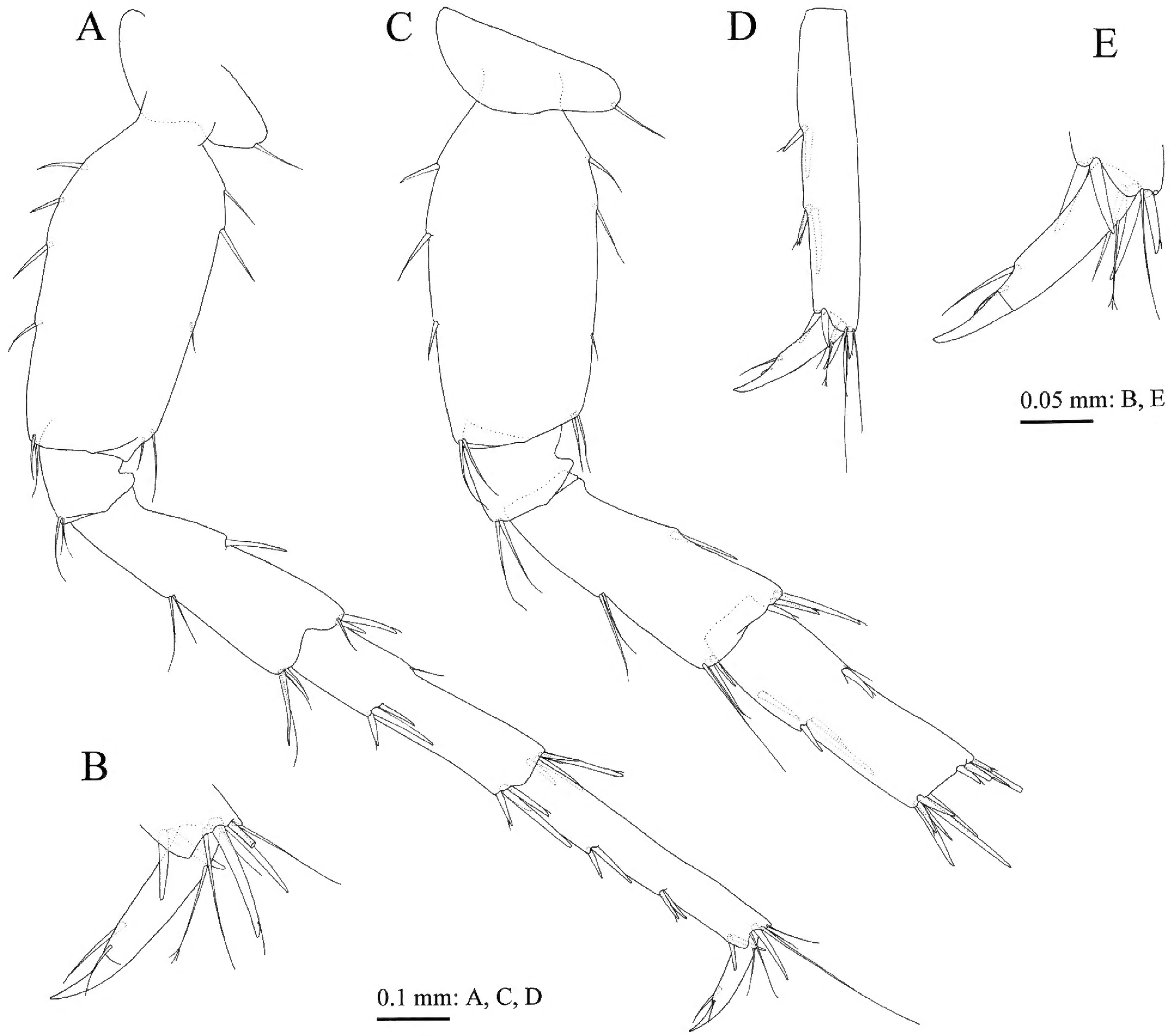

Figure 6. *Pseudocrangonyx gudariensis* Tomikawa & Sato, sp. n., holotype, male (3.9 mm), NSMT-Cr 24603. **A** pereopod 6, lateral view; **B** dactylus of pereopod 6, lateral view; **C** coxa–carpus of pereopod 6, lateral view; **D** propodus–dactylus of pereopod 6, lateral view; **E** dactylus of pereopod 7, lateral view.

Telson (Fig. 7O) length 1.2 times as long as wide, cleft for 0.08 times of length, each telson lobe with 2 lateral long penicillate setae, 2 apical robust and 1 apical short penicillate setae.

Female [NSMT-Cr 24604, 3.1 mm]. Antenna 1 (Fig. 8A) 0.58 times as long as body length, primary flagellum 12-articulate. Antenna 2 (Fig. 8B) 0.64 times as long as antenna 1, calceoli absent; flagellum 0.64 times as long as peduncular articles 4 and 5 combined, 5-articulate, first 2 of which with 1 robust seta, lacking calceoli.

Lacinia mobilis of left mandible 5-dentate.

Gnathopod 1 (Fig. 8C, D) with coxa width 1.9 times as long as depth; palmar margin (Fig. 8D) with 4 medial and 4 lateral distally notched robust setae. Gnathopod 2 (Fig. 8E, F) with coxa width 1.8 times as long as depth; palmar margin (Fig. 8F) with 9 medial and 5 lateral robust setae, some distally notched.

Brood plates (Fig. 8G) slender, with numerous setae, on gnathopod 2 and pereopods 3–5.

Uropod 1 (Fig. 9A) with 3 robust setae on inner margin of inner ramus, basal part with 2 slender setae; outer ramus 0.80 times as long as inner. Uropod 2 (Fig. 9B) with 6 simple robust setae and 1 slender seta on distal part of inner ramus. Uropod 3 (Fig. 9C) with peduncle 0.32 times as long as outer ramus; terminal article of outer ramus 0.35 times as long as proximal article.

Etymology. The specific name is an adjective derived from Gudari-numa, the type locality of the new species.

Distribution and habitat. This species is known only from the type locality. The specimens were collected from interstitial water in the gravelly bottom. Water temperature of the habitat was stable and around 7°C throughout the year (Baba and Ohtaka unpublished).

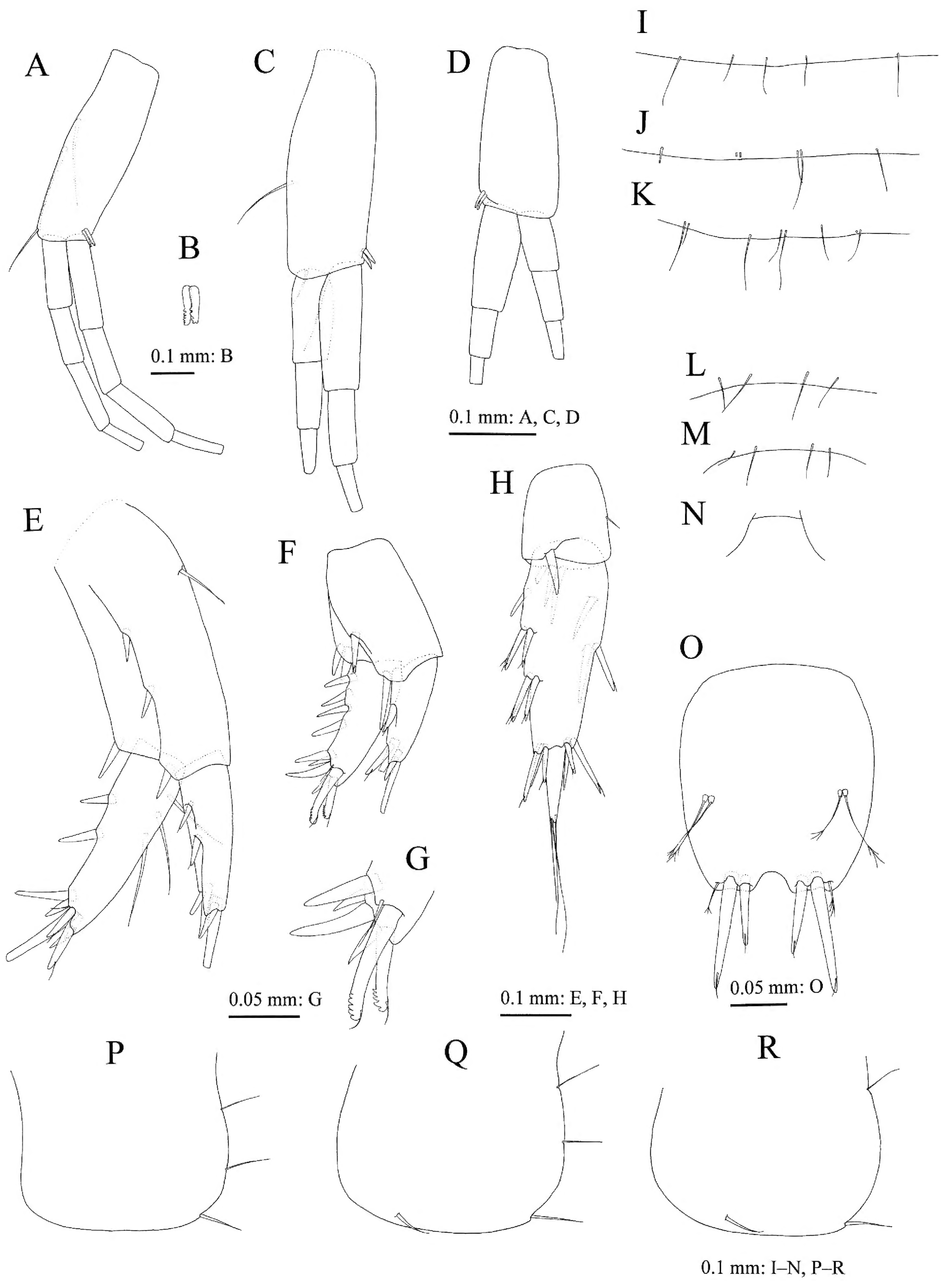

Figure 7. *Pseudocrangonyx gudariensis* Tomikawa & Sato, sp. n., holotype, male (3.9 mm), NSMT-Cr 24603. **A** pleopod 1, anterior view; **B** retinacula on peduncle of pleopod 1, anterior view; **C** pleopod 2, anterior view; **D** pleopod 3, anterior view; **E** uropod 1, dorsal view; **F** uropod 2, dorsal view; **G** distal part of inner ramus of uropod 2, dorsal view; **H** uropod 3, dorsal view; **I–K** dorsal margins of pleonites 1–3, respectively, dorsal views; **L–N** dorsal margins of urosomites 1–3, respectively, dorsal views; **O** telson, dorsal view; **P–R** epimeral plates 1–3, respectively, lateral views.

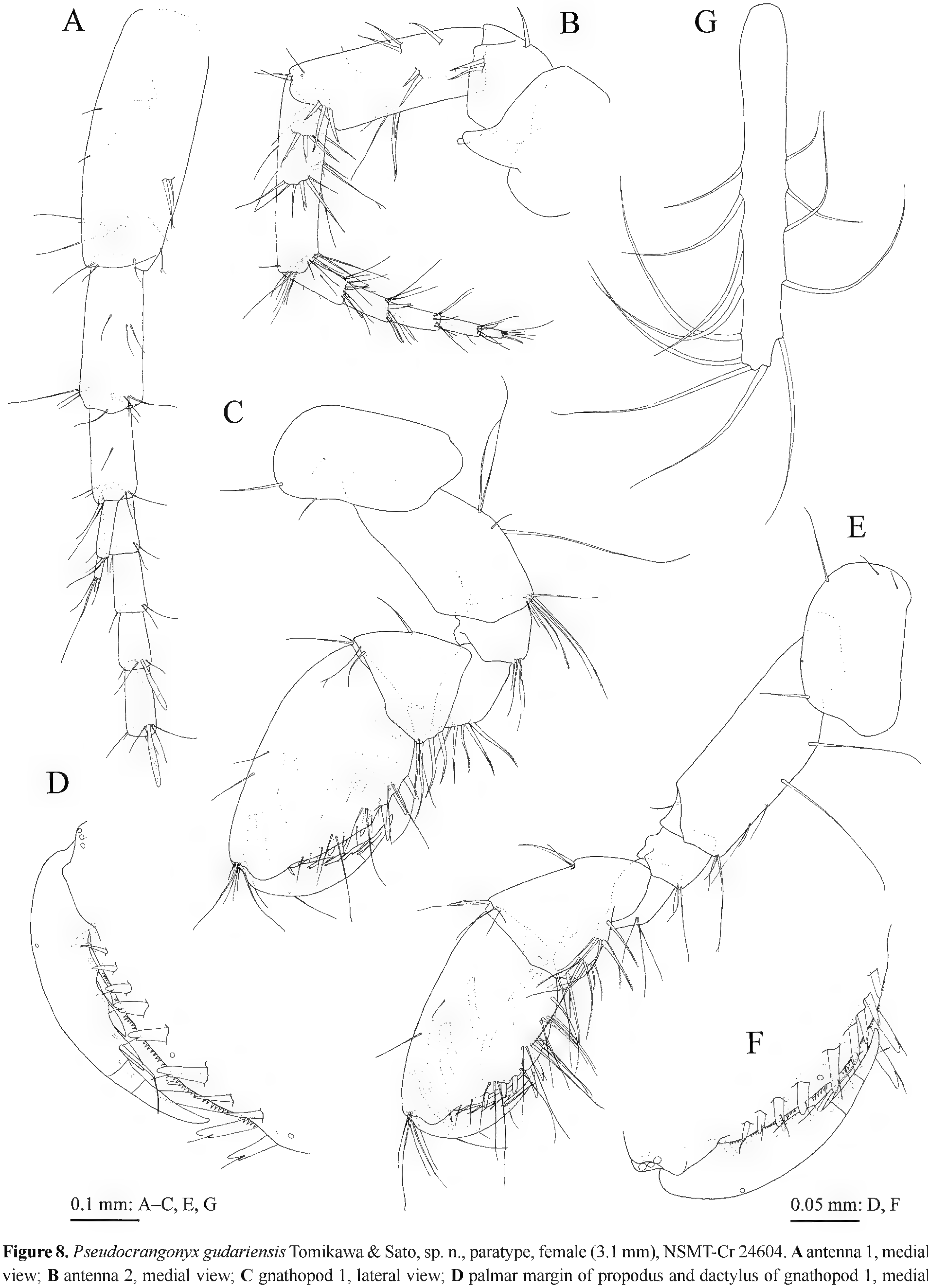

Figure 8. *Pseudocrangonyx gudariensis* Tomikawa & Sato, sp. n., paratype, female (3.1 mm), NSMT-Cr 24604. **A** antenna 1, medial view; **B** antenna 2, medial view; **C** gnathopod 1, lateral view; **D** palmar margin of propodus and dactylus of gnathopod 1, medial view; **E** gnathopod 2, lateral view; **F** palmar margin of propodus and dactylus of gnathopod 2, medial view.

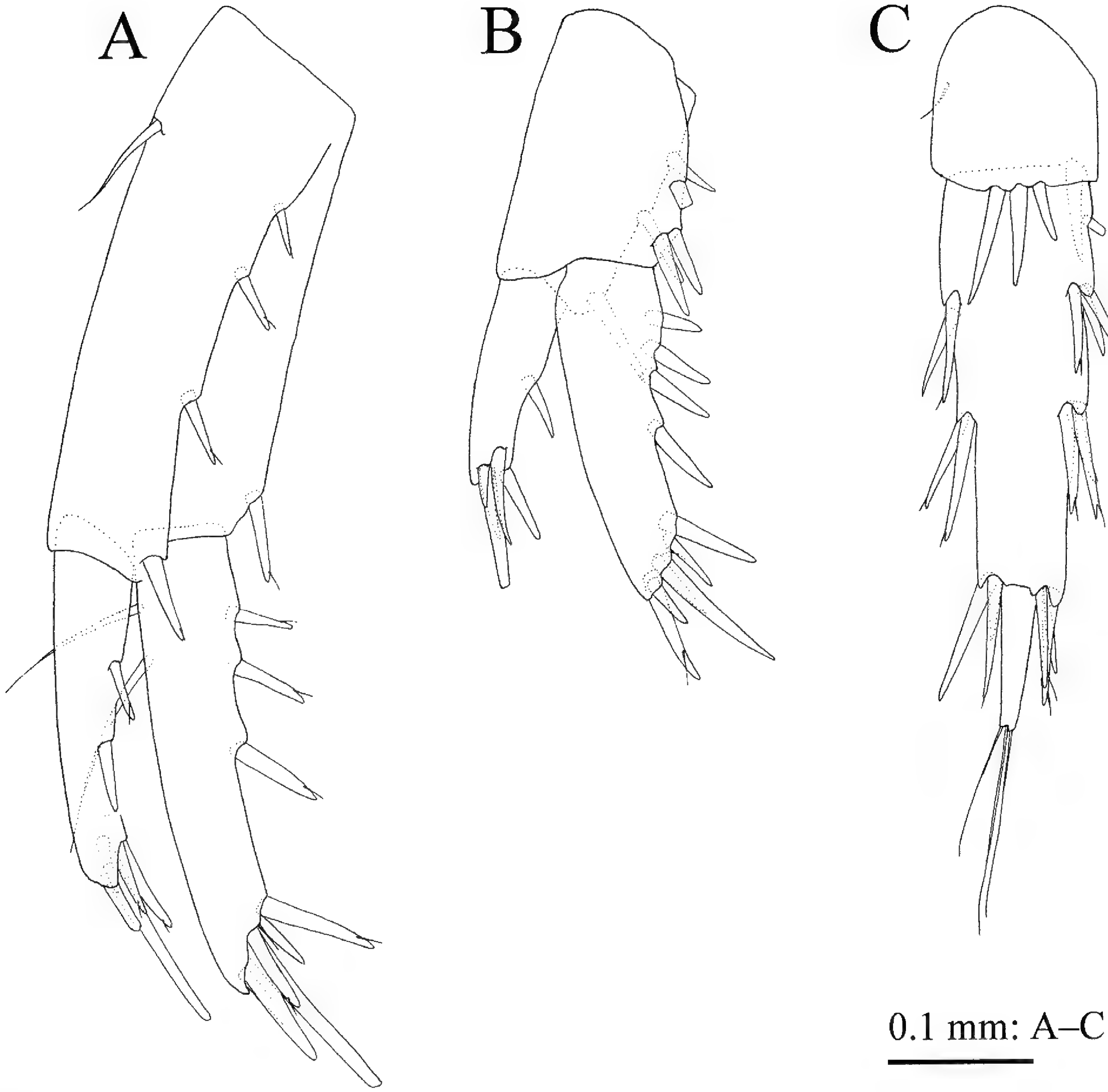

Figure 9. *Pseudocrangonyx gudariensis* Tomikawa & Sato, sp. n., paratype, female (3.1 mm), NSMT-Cr 24604. **A–C** uropods 1–3, respectively, dorsal views.

Remarks. *Pseudocrangonyx gudariensis* is morphologically similar to *P. coreanus* described from the Korean Peninsula. The deposited female paratypes of the latter species have calceoli on antenna 2 and pleopods without bifid setae on inner basal margin of inner ramus, which are features that were not mentioned in the original description (NSMT-Cr 13521–13522; Tomikawa and Onodera, personal observation).These two species share the following features: 1) relatively small body size (smaller than 6 mm), 2) eye completely absent, 3) carpus of gnathopod 2 without serrate robust setae on posterodistal corners, 4) outer margin or outer distal corner of pleopods 1 and 2 with setae, 5) inner basal margin of inner ramus of pleopods without bifid setae, and 6) small number of articles (less than 5) of rami of pleopods. However, *P. gudariensis* is distinguished from *P. coreanus* by the following features (features of *P. coreanus* in parentheses): 1) antenna 2 of female without calceoli (present); 2) palmar margins of gnathopods 1 and 2 with distally notched robust setae (absent); 3) inner margin of inner ramus of uropod 2 with 4 (0 or 1) robust setae; and 4) basal part of inner ramus of uropod 2 without slender seta (present).

Pseudocrangonyx gudariensis is also similar to *P. febras* from river basin of Primorye, Russia in having 1) relatively small body size (smaller than 6.5 mm), 2) eye completely absent, 3) palmar margins of gnathopods 1 and 2 with distally notched robust setae, 4) small number of articles (less than 6) of rami of pleopods, and 5) urosomite 1 without basal setae. However, *P. gudariensis* is distinguished from the latter by the following features (features of *P. febras* in parentheses): 1) carpus of gnathopod 2 without serrate robust setae on posterodistal cor-

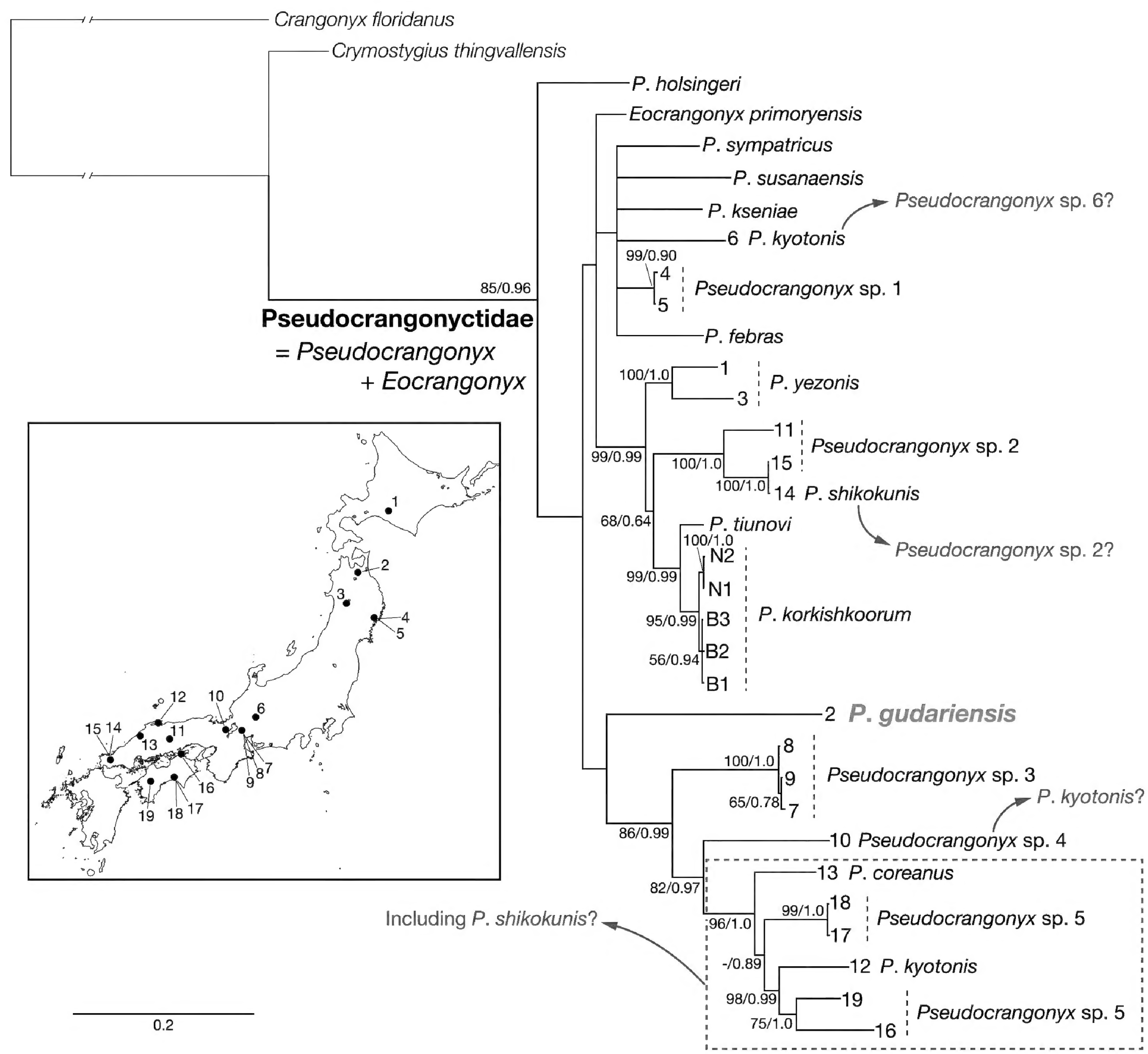

Figure 10. Bayesian inference tree for 2,397 bp of nuclear 28S rRNA plus histone H3 and mitochondrial COI and 16S rRNA markers, with the map modified from Fig. 1. Numbers on nodes represent bootstrap values for maximum likelihood and Bayesian posterior probabilities. Specimen numbers are also shown in Fig. 1 and Table 1.

ners (present), 2) peduncle of pleopods 1 and 2 with setae (absent), and 3) article 2 of uropod 3 longer (shorter) than setae on distal part of article 1.

Molecular phylogenies

The obtained BI tree (Fig. 10) had an almost identical topology to that of the ML tree (ln $L = -13551.11$; not shown). The monophyly of the family Pseudocrangonyctidae (*Pseudocrangonyx* + *Eocrangonyx*) was well recovered (BS = 88%, PP = 0.96). However, our analyses failed to obtain robust phylogenetic relationships of the known *Pseudocrangonyx* species, and to determine the precise phylogenetic positions of each of the new species *P. gudariensis* (sample #2), *P. kyotonis* sensu Nunomura (1975) from Gifu (#6), and unidentified *Pseudocrangonyx* amphipods from Iwate (#4, 5). Two monophyletic lineages received high support values. The one lineage (BS = 99%, PP = 0.99) contained *P. yezonis* clade (BS = 99%, PP = 1.0; #1, 3), the clade (BS = 100%, PP = 1.0) of the *Pseudocrangonyx* species from Okayama (11) and Yamaguchi (#14, 15) including *P. shikokunis* sensu Uéno (1927) (#14), and the clade (BS = 100%, PP = 1.0) comprised two Russian species, *P. korkishkoorum* and *P. tiunovi*. However, the relationships among these three clades were unresolved.

The other monophyletic lineage (BS = 93%, PP = 0.99) consisted of unidentified *Pseudocrangonyx* species from Shiga Prefecture (#7–10), and from Shikoku (#16–19), plus *P. coreanus* sensu Narahara et al. (2009) from Shimane (#10), and *P. kyotonis* sunsu Uéno (1971a) from Shimane as well (#12). Three specimens from Shiga (#7–9) formed a well supported clade (BS = 100%, PP = 1.0). This subclade was a sister lineage to the clade

(BS = 88%, PP = 0.97) comprised reminders including a specimen from Shiga (#10). Specimens from Shimane (#12, 13) and Shikoku region (#16–19) formed a monophyletic lineage (BS = 96%, PP = 1.0). Monophyly of the *Pseudocrangonyx kyotonis* sensu Uéno (#12) and two specimens from Kagawa (#16) and Ehime (#19) was well supported (BS = 98%, PP = 0.99); the latter two (#16, 19) formed a clade, but this relationship was not strongly supported in the ML analysis (BS = 69%, PP = 1.0).

Discussion

As mentioned in the Remarks, *P. gudariensis* is morphologically similar to *P. coreanus* and *P. febras*. These three species share the following characteristics: relatively small body size, absence of basal setae on urosomite 1, and small number of articles of rami of pleopods. However, our phylogenetic analyses failed to recover monophyly of *P. gudariensis* + *P. febras*. In addition, *P. coreanus* sensu Narahara et al. (2009) is genetically distant from *P. gudariensis* and *P. febras*. Therefore, the aforementioned shared characteristics do not reflect phylogenetic relationships of *Pseudocrangonyx* species. Whatever the case, the results of our morphological examination and molecular phylogenetic analyses fully support the distinct taxonomic status of the present new species in this genus.

Sidorov and Holsinger (2007) suggested that the colonization events of the ancestral *Pseudocrangonyx* species to the Japanese Archipelago could have taken place twice: in the Middle–Late Miocene and the Early Pleistocene through land bridges between the continental China and the Japanese Archipelago. The obtained phylogeny also indicated that multiple colonization events of *Pseudocrangonyx* amphipods to the Japanese Archipelago occurred. The present Japanese specimens were split into two clades, and three distinctive lineages, of which phylogenetic positions still remain uncertain. Moreover, the obtained tree showed that the phylogenetic positions of the several Japanese individuals did not reflect their geographical distributions. The specimens distributed in the Chugoku region (#11, 14, 15) did not form a monophyletic group with geographically close samples (e.g., #12, 13). These three specimens formed a clade with the northern Japanese *P. yezonis* and two Russian species. These results indicate that Japanese *Pseudocrangonyx* species experienced a complicated biogeographical history. To clarify the origin and dispersal routes of *Pseudocrangonyx* amphipods, comprehensive taxa sampling and more detailed genetic data are needed.

Our phylogenetic results also revealed that the species diversity of Japanese *Pseudocrangonyx* is quite high, and they should be classified into the known three species and additional undescribed species. Therefore, taxonomic studies should be conducted to determine the systematic accounts of these undescribed amphipods. First, however, the taxonomic status of the three known species described by Akatsuka and Komai (1922) should be revisited. In the obtained tree, *P. kyotonis* sensu Nunomura (1975) (#6) and sensu Uéno (1971a) (#12) were not monophyletic. The latter was genetically more closely related to the *Pseudocrangonyx* species distributed in Shikoku (e.g., #16, 17), where the type locality of *P. shikokunis* is located. Alternatively, *P. shikokunis* sensu Uéno (1927) (#14) was distantly related to the *Pseudocrangonyx* specimens collected in Shikoku. These results highlighted that the previous studies of *Pseudocrangonyx* in Japan contained misidentified or taxonomically uncertain records.

In addition to *P. gudariensis*, *P. kyotonis* sensu Uéno (1971a), *P. kyotonis* sensu Nunomura (1975), and *P. coreanus* sensu Narahara et al. (2009), five unidentified Japanese *Pseudocrangonyx* species were identified based on molecular phylogenetic analyses (Fig. 10). Among these five species, it is highly possible that *Pseudocrangonyx* sp. 5 and *Pseudocrangonyx* sp. 4 represent the true *P. shikokunis* and *P. kyotonis*, respectively, because their collection localities are close to each of the type localities of these species. Accordingly, *P. kyotonis* sensu Nunomura (1975) and *P. shikokunis* sensu Uéno (1927) could be considered unidentified *Pseudocrangonyx* sp. 6 and *Pseudocrangonyx* sp. 2, respectively. To confirm whether these taxonomic treatments are adequate, molecular phylogenetic analyses including topotypic sequences of described species and detailed morphological analyses are needed.

Our phylogenetic analyses also shed light onto the taxonomic account of *Eocrangonyx* Schellenberg, 1936. This genus have been placed under the family Pseudocrangonyctidae along with *Pseudocrangonyx* (Holsinger 1989). These two genera bear a close resemblance to each other in general morphology. *Eocrangonyx* is distinguished from *Pseudocrangonyx* by the absence of article 2 of uropod 3 (Holsinger 1989). However, Sidorov and Holsinger (2007) revealed the presence of extremely reduced article 2 of uropod 3 in the Russian *E. stygoedincus* (Sidorov and Holsinger, 2007). Subsequently, Tomikawa and Shinoda (2016) also revealed the same characteristics in *E. japonicus* (Uéno, 1930), which is the type species of *Eocrangonyx*. Our phylogenetic tree showed that *Pseudocrangonyx* and *Eocrangonyx* are phylogenetically closely related (Fig. 10). However, the phylogenetic position of *E. primoryensis* remains unresolved. In addition, *E. japonicus* genetic data have been never assessed. To evaluate the independence of *Pseudocrangonyx* and *Eocrangonyx*, and the validity of article 2 of uropod 3 as a generic diagnostic character, it is necessary to clarify the phylogenetic relationships between *Pseudocrangonyx* and *Eocrangonyx* by including additional species and genetic markers. Consequently, there will be an enhanced understanding of species diversity and evolutionary history of the Far Eastern subterranean Crangonyctoidea species.

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